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Involvement of Multiple Efflux Transporters in Hepatic Disposition of Fexofenadine

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ABSTRACT

Fexofenadine (FEX) is mainly eliminated from the liver into bile in unchanged form. We demonstrated previously that organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 are involved in the hepatic uptake of FEX. However, little is known about the mechanisms controlling the hepatic efflux of FEX from the liver to bile and blood. In the present study, the involvement of hepatic efflux transporters in the pharmacokinetics of FEX was investigated in both in vitro and in vivo studies. Vectorial transport of FEX was observed in OATP1B3/human bile salt export pump (hBSEP) double transfectants but not in OATP1B3/human breast cancer resistance protein double transfectants, which indicates the possible contribution of hBSEP to the biliary excretion of FEX in humans. In multidrug resistance-associated protein 2 (Mrp2)$^{-/-}$ mice, the biliary excretion clearance based on the plasma concentration and the liver-to-plasma concentration ratio significantly decreased, whereas the biliary excretion clearance based on the liver concentration decreased only with 20%, suggesting the minimum contribution of Mrp2 to its biliary excretion. ATP-dependent transport of FEX was observed in hMRP3-enriched membrane vesicles but not hMRP4. In Mrp3$^{-/-}$ mice, the biliary excretion clearance based on both the plasma and liver concentration and the liver-to-plasma concentration ratio increased, suggesting the significant contribution of Mrp3 to its sinusoidal efflux and the up-regulation of its biliary excretion in Mrp3$^{-/-}$ mice. On the other hand, pharmacokinetics of FEX remained unchanged in Mrp4$^{-/-}$ mice. This information provides a novel insight into the transporters important for FEX disposition.

Fexofenadine (FEX) is an orally active nonsedating histamine H_1 receptor antagonist that is prescribed for oral treatment of allergic rhinitis and chronic idiopathic urticaria. After oral administration of [^{14}C]FEX to healthy volunteers, 80% of the dose was recovered in feces and 12% in urine, in unchanged form (Lippert et al., 1995). Because the absolute oral bioavailability of FEX is reported to be 33% (product information, Hoechst Marion, Roussel, Laval, Quebec, Canada), it follows that two-thirds of the bioavailable FEX is excreted into bile. Therefore, hepatic transport of FEX is one of the determinants for its systemic clearance.

We demonstrated previously that human organic anion transporting polypeptide 1B1 (hOATP1B1/SLCO1B1) and hOATP1B3 (SLCO1B3) contribute to the hepatic uptake of FEX in humans (Shimizu et al., 2005; Matsushima et al., in press). On the other hand, the transporters involved in its biliary excretion have not been clarified yet. In the canalicular membrane, several ATP binding cassette transporters such as multidrug resistance-associated protein 2 (MRP2/ABCC2), P-glycoprotein/multidrug resistance 1 (P-gp/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and bile salt export pump (BSEP/ABCB11) are involved in the excretion of several compounds. MRP2 is responsible for the biliary excretion of a wide variety of organic anions, including glutathione and glucuronide conjugates and drugs such as pravastatin (Suzuki and Sugiyama, 1998). MDR1 preferentially accepts hydrophobic cationic and neutral compounds (Hoffmann and Kroemer, 2004), whereas BCRP accepts various kinds of organic anions (Suzuki et al., 2003; van

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ABBREVIATIONS: FEX, fexofenadine; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CL, clearance; EG, estradiol-17β-D-glucuronide; LUI, liver uptake index; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein; TC, taurocholate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GFP, green fluorescent protein; HEK, human embryonic kidney; LC/MS, liquid chromatography/mass spectrometry.

Herwaarden et al., 2003; Hirano et al., 2005b; Merino et al., 2005). Although BSEP was believed to accept only bile salts (Byrne et al., 2002), recent studies indicate that BSEP transports some drugs such as vinblastine and pravastatin (Lecureur et al., 2000; Hirano et al., 2005a).

It has been shown that FEX is a substrate of P-gp and hMRP2 (Cvetkovic et al., 1999; Matsushima et al., in press), whereas nobody has checked whether FEX is a substrate of hBCRP and hBSEP. Tahara et al. (2005) investigated biliary excretion of FEX using Eisai hyperbilirubinemic rats (Mrp2-deficient rats) and Mdr1a/1b$^{-/-}$ and Bcrp1$^{-/-}$ mice. We were surprised to find that these transporters didn't have an effect on FEX biliary excretion clearance based on the liver concentration (Tahara et al., 2005). These results suggest that the biliary excretion of FEX is mediated by unknown transporters distinct from rat Mrp2 (rMrp2), mouse Mdr1a/1b (mMdr1a/1b), and mBcrp1. However, there may be a species difference in the mechanisms of FEX biliary excretion between rats and mice. Mrp2$^{-/-}$ mice have been established, and the impact of mMrp2 on the pharmacokinetics of some drugs and toxins has been characterized (Chu et al., 2006; Vlaming et al., 2006). Therefore, to clarify the biliary excretion mechanisms of FEX in greater detail, we investigated whether FEX is accepted by hBCRP and hBSEP/rBsep in in vitro studies and demonstrated the importance of mMrp2 in its biliary excretion using Mrp2$^{-/-}$ mice.

On the other hand, it has become clear that MRP3 (ABCC3) and MRP4 (ABCC4) are important transporters in sinusoidal efflux (Borst et al., 2007). MRP3 can transport a wide variety of organic anions, such as glucuronides, glutathione conjugates, bile acids, and methotrexate (Hirohashi et al., 1999, 2000; Kool et al., 1999; Zelcer et al., 2001; Zeng et al., 2001). Because rMrp3 is expressed at low levels in normal rat liver and its expression markedly increases in Eisai hyperbilirubinemic rats (Hirohashi et al., 1998), the physiological role of rMrp3 has been believed to be the protection of hepatocytes from intrahepatic toxins such as bile acids only under pathological conditions (e.g., cholestasis). Recent in vivo studies using Mrp3$^{-/-}$ mice suggest that mMrp3 contributes to sinusoidal efflux of various glucuronide conjugates (Borst et al., 2007). The substrate specificity of MRP4 overlaps with that of MRP3, but it is somewhat distinguished from MRP3 by its ability to transport nucleotide analogs (van Aubel et al., 2002; Reid et al., 2003). The physiological function of MRP4 in hepatocytes is considered to protect hepatocytes from bile acids under cholestatic conditions as if it is up-regulated during cholestasis (Keitel et al., 2005; Mennone et al., 2006). Because both hMRP3 and hMRP4 are expressed in human liver under physiological conditions (Konig et al., 1999; Rius et al., 2003), these transporters may also be involved in the hepatic distribution of drugs. Therefore, we investigated the role of MRP3 and MRP4 in the sinusoidal efflux of FEX using hMRP3- and hMRP4-enriched membrane vesicles and Mrp3$^{-/-}$ and Mrp4$^{-/-}$ mice.

Materials and Methods

Materials

[^{3}H]Estradiol-17β-D-glucuronide (EG; 45 Ci/mmol) and [^{3}H]taurocholate (TC; 3.5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]Pitavastatin (44.6 Ci/mmol) was donated by Kowa Co. Ltd. (Tokyo, Japan). FEX hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). All other chemicals and reagents were of analytical grade and were commercially available.

Animals

Male FVB mice (wild type) and Mrp2$^{-/-}$ mice were described previously (Vlaming et al., 2006). Male FVB mice (wild type) and Mrp3$^{-/-}$ mice were kindly donated by Dr. P. Borst (Division of Molecular Biology and Cancer of Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, the Netherlands) (Zelcer et al., 2006). Male C57BL/6 mice (wild type) and Mrp4$^{-/-}$ mice were kindly donated by Dr. J. D. Schuetz (Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN) (Leggas et al., 2004). All animals were maintained under standard conditions with a reverse dark-light cycle and were treated humanely. Food and water were available ad libitum. All of the animal studies performed in this article were approved by the Institutional Animal Care Committee and carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

Cell Culture

hOATP1B3-expressing MDCKII cells and vector-transfected control cells used in this study were constructed previously (Ishiguro et al., in press). Transporter-expressing, vector-transfected MDCKII or parent HEK293 cells were grown in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic solution (Sigma-Aldrich) at 37°C under 5% CO_2 and 95% humidity.

Construction of hBSEP- and hBCRP-Expressing Cells

To construct MDCKII cells expressing hBSEP and hBCRP, MDCKII cells were infected with recombinant adenovirus harboring hBSEP and hBCRP cDNA at a multiplicity of infection of 150, 48 h before all experiments. The virus titer was determined as described previously (Hayashi et al., 2005).

Transcellular Transport Study

The transcellular transport study was performed as reported previously (Matsushima et al., 2005). In brief, MDCKII cells were grown on Transwell membrane inserts (6.5 mm diameter, 0.4 μm pore size; Corning Costar, Bodenheim, Germany) at confluence for 7 days, and the expression level of transporters was induced by the replacement of culture medium with that supplemented with 5 mM sodium butyrate 48 h before the transport study. Cells were first washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4) at 37°C. Thereafter, Krebs-Henseleit buffer containing substrates was added either to the apical compartments (250 μl) or to the basolateral compartments (1 ml). After a designated period, 50 μl of medium was taken from the opposite side to the added substrate. When using FEX as a substrate, 50-μl aliquots were used for LC/MS quantification as described below. When using [^{3}H]pitavastatin as a substrate, the radioactivity in the sample was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter, Fullerton, CA). At the end of the experiments, cells were washed with ice-cold Krebs-Henseleit buffer and solubilized in 500 μl of 0.2 N NaOH. After the addition of 100 μl of 1 N HCl, 50-μl aliquots were used to determine protein concentrations by the method of Lowry with bovine serum albumin as a standard.

Transport Studies with Membrane Vesicles

Membrane vesicles were prepared from human BSEP-, rat Bsep-, human MRP3-, and human MRP4-transfected HEK293 cells according to the method described previously (Hayashi et al., 2005; M. Hirouchi, H. Kusuhara, and Y. Sugiyama, unpublished observations). The transport studies were performed using a rapid filtration

technique. In brief, 15 μl of transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.4) containing FEX, EG, or TC was preincubated at 37°C for 3 min and then rapidly mixed with 5 μl of membrane vesicle suspension (10-μg time course study or 15 μg of protein for saturation study). The reaction mixture contained 5 mM ATP or AMP, along with the ATP-regenerating system (10 mM creatinine phosphate and 100 μg/μl creatinine phosphokinase). The transport reaction was terminated by the addition of 1 ml of ice-cold stop solution (containing 10 mM Tris-HCl, 250 mM sucrose, and 0.1 N NaCl, pH 7.4). The reaction mixture was passed through a 0.45-μm HA filter (Millipore Corporation, Billerica, MA) and then washed twice with 5 ml of stop solution. FEX retained on the filter was then quantified by LC/MS as described below. In the case of [^{3}H]EG and [^{3}H]TC, filters with trapped membrane vesicles were mixed with scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan), and the radioactivity retained on the filter was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter).

Intravenous Constant Infusion Studies in Mice

Mice weighing approximately 24 to 32 g were used throughout the experiments. Under anesthesia with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical, Osaka, Japan), the jugular vein was cannulated with a polyethylene catheter (PE-10; BD Biosciences, Sparks, MD) for the injection of FEX. The bile duct was cannulated with a Teflon tube (UT-3; Unique Medical, Tokyo, Japan) for bile collection, and the urinary bladder was cannulated with a Teflon tube (industrial use) for urine collection. The mice received a constant infusion of FEX at a dose of 623 to 804 nmol/h/kg of body weight for 180 min (Harvard Apparatus syringe infusion pump; Harvard Apparatus Inc., Holliston, MA). Because mice were anesthetized throughout the experiment, they were kept warm with a hot plate for experimental animals (Natsume Seisakusyo, Tokyo, Japan). Bile and urine were collected in preweighed test tubes at 20-min intervals throughout the experiment. Blood samples (approximately 30 μl) were collected from the jugular vein at 120, 140, 160, and 180 min after starting the infusion. Plasma was prepared by centrifuging the blood samples (3000*g*). The mice were sacrificed after 180 min, and the entire liver, kidney, and brain were excised immediately. The tissues were weighed and stored at −80°C until the assay.

Kinetic Analyses in the Infusion Study The steady-state plasma concentration (C_{ss}) was assessed as the mean plasma concentration at 120, 140, 160, and 180 min, whereas the steady-state liver (C_{liver}), kidney (C_{kidney}), and brain (C_{brain}) concentrations were determined at 180 min. The total plasma clearance ($CL_{tot,plasma}$) was obtained by dividing the infusion rate by C_{ss}. The biliary and urinary clearances ($CL_{bile,plasma}$, $CL_{urine,plasma}$) gave the mean clearance values calculated by dividing the biliary and urinary excretion rates (V_{bile}, V_{urine}) by C_{ss}. $CL_{bile,liver}$ was the biliary clearance based on C_{liver}. The $K_{p,liver}$, $K_{p,kidney}$, and $K_{p,brain}$ represented the ratio of C_{liver}, C_{kidney}, and C_{brain} to C_{ss}, respectively.

Liver Uptake Index Study

Under anesthesia with pentobarbital sodium (Nembutal), the portal vein of male FVB and Mrp3$^{-/-}$ mice (weighing 28–32 g) was cannulated with polyethylene tubing (PE-10). FEX dissolved in mouse plasma was rapidly injected into the portal vein. At 17 s after the bolus administration of FEX (10 nmol/kg of body weight), which is long enough for the bolus to pass completely through the liver but short enough to prevent recirculation of the compound, the portal vein, hepatic artery, and bile duct were cut, and the liver was excised. The tissue was weighed and stored at −80°C until assay.

LC/MS Analyses

Sample Pretreatment. The aliquots (50 μl) obtained from the transcellular transport study were precipitated with 200 μl of methanol containing 10 nM midazolam as an internal standard. After centrifugation (15,000*g*, 10 min, 4°C) of the mixture, 50 μl of 0.05% formic acid was added to 50 μl of supernatant. In the membrane vesicle studies, FEX retained on the filter was recovered in 1 ml of methanol containing 1 nM midazolam as an internal standard by sonication for 15 min. After centrifugation, the supernatants (750 μl) were evaporated using a centrifugal concentrator (CC-105; TOMY, Tokyo, Japan), and dissolved in 100 μl of mobile phase (see LC/MS Instrumentation and Operating Conditions). Plasma (5 μl) obtained from the infusion study was mixed with 15 μl of 0.05% volume of formic acid and precipitated with methanol (80 μl) containing midazolam (50 nM) as an internal standard. Bile (2 μl) obtained from the infusion study was mixed with 48 μl of 0.05% volume of formic acid. Then, 90 μl of 0.05% volume of formic acid was added to 10 μl of the mixed solution and precipitated with methanol (250 μl) containing the internal standard. Urine (10 μl) obtained from the infusion study was precipitated with methanol (500 μl) containing midazolam (internal standard). Liver, kidney, and brain obtained from the infusion study or the liver uptake index (LUI) study were added to a 3-fold volume of PBS and homogenized with a handy-type homogenizer (Multipro 395; Dremel Corporation, Racine, WI). Homogenate (50 μl) obtained from the liver and kidney was precipitated with methanol (750 μl) containing midazolam (internal standard) and then centrifuged, and the supernatant was diluted with an equal volume of 0.05% volume of formic acid. Homogenate (50 μl) obtained from the brain was precipitated with methanol (500 μl) containing 5 nM midazolam as an internal standard and centrifuged, and then the supernatant (400 μl) was evaporated using a centrifugal concentrator (CC-105) and dissolved in 80 μl of mobile phase. The obtained samples were subjected to the LC/MS analysis to determine the concentration of FEX.

LC/MS Instrumentation and Operating Conditions. An LC/MS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. The samples were separated on a CAPCELL PAK C18 MG column (3 μm, 4.6 mm internal diameter, 75 mm; Shiseido, Tokyo, Japan) in binary gradient mode. The mobile phase consisted of 0.05% formic acid and methanol. The methanol concentration was initially 48% and then was linearly increased up to 61.5% over 4.5 min. Finally, the column was re-equilibrated at a methanol concentration of 48% for 3 min. The total run time was 7.5 min. FEX and midazolam were eluted at 4.1 and 2.8 min, respectively. In the mass analysis, FEX and midazolam were detected at a mass-to-charge ratio of 502.3 and 326.1 under positive ionization conditions. The interface voltage was 3.5 kV, and the nebulizer gas (N_2) flow was 1.5 L/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

Quantification of mRNA and Protein Expression Levels of the Hepatic Transporters in Mice

Total RNA was isolated from the livers of three wild-type FVB mice and Mrp3$^{-/-}$ mice using ISOGEN (Nippon Gene, Tokyo, Japan) and converted to cDNA using a random primer. Real-time quantitative PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions. The primers used in the quantification are listed in Table 1. G3pdh was used as a housekeeping gene for the internal standard. An external standard curve was generated by dilution of the target PCR product, which was purified by agarose gel electrophoresis. The absolute concentration of external standard was measured by PicoGreen dsDNA Quantification Reagent (Invitrogen). To confirm the amplification specificity, PCR products were subjected to a melting curve analysis and gel electrophoresis. All gene expressions in each reaction were normalized by the expression of G3pdh in the same sample.

For Western blot analyses, crude membrane was prepared from the livers of five wild-type FVB and Mrp3$^{-/-}$ mice according to the method used in the previous report (Niinuma et al., 1999). After the crude membrane was suspended in PBS, it was frozen in liquid N_2 and stored at −80°C until used. The protein concentration in the

crude membrane vesicles was determined by the method of Lowry with bovine serum albumin as a standard. The membrane fraction was dissolved in 3× SDS sample buffer (New England Biolabs, Ipswich, MA) and loaded on to a 7 or 12.5% SDS-polyacrylamide electrophoresis gel with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England Biolabs). Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad Laboratories, Hercules, CA) at 15 V for 1 h. The membrane was blocked with PBS containing 5% skimmed milk overnight at 4°C. After washing with Tris-buffered saline with 0.05% Tween 20, the membrane was incubated at room temperature in PBS containing 5% skimmed milk with 125-fold diluted anti-Mrp2 monoclonal antibody (M_2III-6; Alexis, Gruenberg, Germany) for 2 h, 100-fold diluted anti-Mdr1 monoclonal antibody (C219; Signet, Dedham, MA) for 1 h, 200-fold diluted anti-Bcrp monoclonal antibody (BXP-53; Signet) for 2 h, 500-fold diluted anti-Bsep polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h, 1000-fold diluted anti-Mrp3 polyclonal antibody for 2 h (Akita et al., 2002), 100-fold diluted anti-Mrp4 monoclonal antibody (M_4I-10; Abcam, Cambridge, UK) for 1 h, or 1000-fold diluted anti-mouse β-actin monoclonal antibody (Millipore) for 2 h. For the detection of mMrp2, mMdr1, mBcrp, mMrp4, and mβ-actin, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 goat anti-mouse IgG (Invitrogen) for 1 h. For the detection of mMrp3, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 goat anti-rabbit IgG (Invitrogen). For the detection of mBsep, the membrane was placed in contact with 1000-fold diluted Alexa Flour 680 donkey anti-goat IgG (Invitrogen). The fluorescence was assessed in a densitometer (Odessey; ALOKA, Tokyo, Japan).

TABLE 1
Nucleotide sequences of the primers used in real-time quantitative PCR

Transporter	Forward Primer	Reverse Primer
Oatp1a1	cagataaatggatttgccag	gtcaacaaatagttacagag
Oatp1a4	atagcttcaggcgcatttac	ttctccatcattctgcatcg
Oatp1b2	ttcaccacaacaatggccta	ttttccccacagacaggttc
Mrp2	tctctggtttgcctgtta	gcagaagacaatcaggttt
Mrp3	gctctcacaaggtggtacaa	caggttgaaacaggcactca
Mrp4	gatcgcctacgtttctcagc	ccggtctcctataaccgtca
Mdr1a	tcattgcgatagctggag	caaacttctgctcccgagtc
Mdr1b	acctgctgttggcgtatttg	ttcctccagactgctgttgc
Bcrp	aaatggagcacctcaacctg	cccatcacaacgtcatcttg
Bsep	aaatcggatggtttgactgc	tgacagcgagaatcaccaag
Mate1	aacaccatctcccagtttgc	gccaaggataccactcagga
G3pdh	tgcgacttcaacagcaactc	cttgctcagtgtccttgctg

Measurement of the Concentration of Total Bile Acids and GSH in the Liver and Bile in FVB Mice and Mrp3$^{-/-}$ Mice. Mice of both strains, weighing approximately 30 g, were used throughout the experiments. Under anesthesia with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical), the bile duct was cannulated with a Teflon tube (UT-3) for bile collection. Bile was collected in preweighed test tubes for a designated time. For the measurement of GSH, bile was collected in tubes filled with 5% metaphosphoric acid-dissolved solution. After collecting bile, the mice were sacrificed, and the entire liver was excised immediately. The liver was weighed,

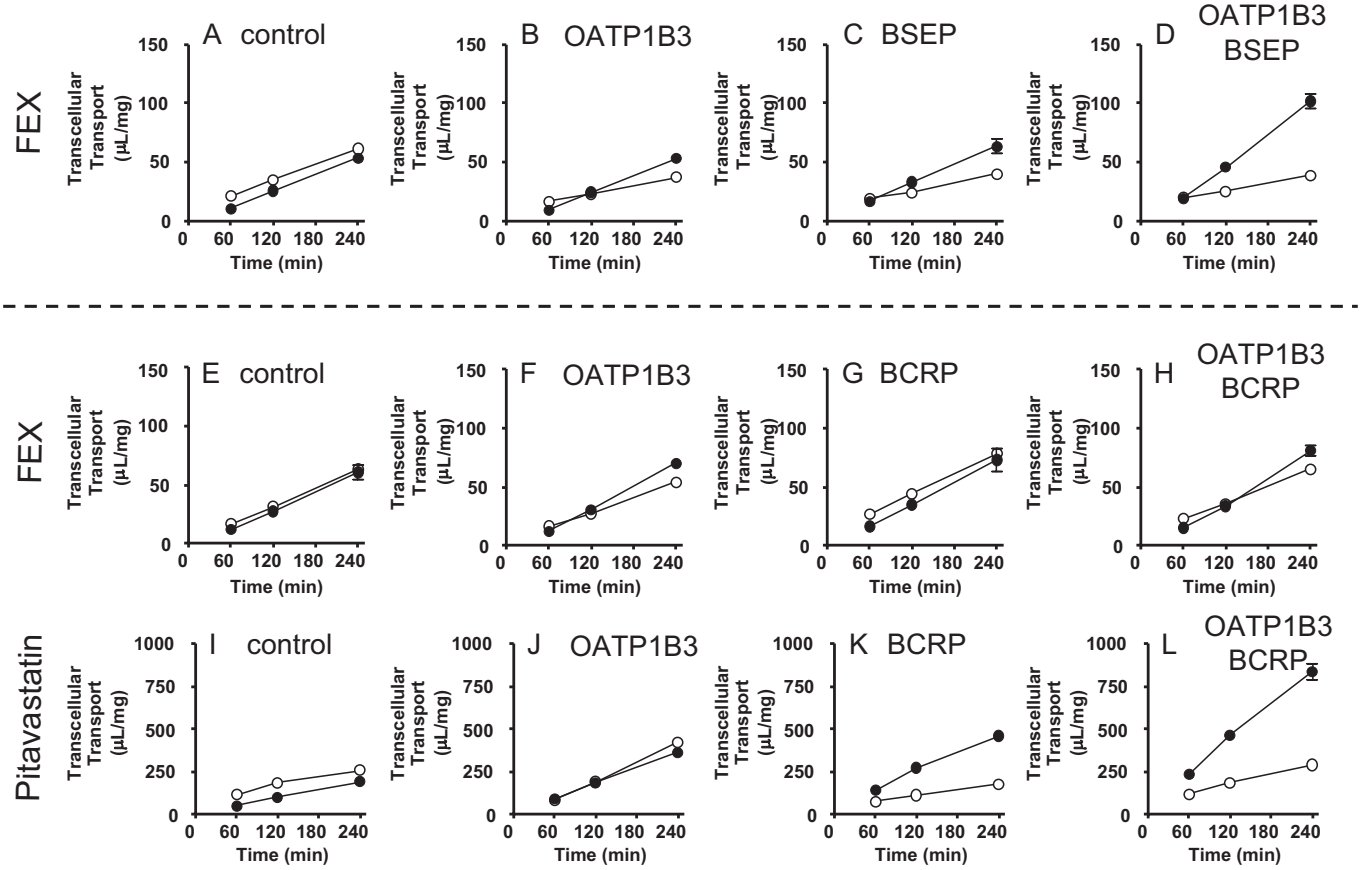

Fig. 1. Time profiles for the transcellular transport of FEX and pitavastatin across hOATP1B3-, hBSEP-, and hBCRP-expressing MDCKII cell monolayers. Transcellular transport of 5 μM FEX (A–H) and 0.1 μM pitavastatin (I–L) across MDCKII cell monolayers expressing hOATP1B3 (B, F, and J), hBSEP (C), hBCRP (G and K), both hOATP1B3 and hBSEP (D), and both hOATP1B3 and hBCRP (H and L) was compared with that across the control MDCKII cell monolayer (A, E, and I). Open and closed circles represent the transcellular transport in the apical-to-basal and basal-to-apical direction, respectively. Each point and vertical bar represent the mean ± S.E. of three determinations. Where no vertical bar is shown, the S.E. was contained within the limits of the symbol.

and a part of it was placed in metaphosphoric acid solution (for the measurement of GSH); the remainder was placed in PBS. Each tissue was homogenized using a handy-type homogenizer (Multipro 395). The concentrations of total bile acids and GSH in bile and liver homogenate were measured using assay kits (total bile acids: Wako, Osaka, Japan; GSH: Oxis, Portland, OR).

Statistical Analyses. Statistical differences were analyzed by using Student's t test to identify significant differences between two sets of data. Significant differences were considered to be present at $p < 0.05$.

Results

Transcellular Transport of FEX and Pitavastatin across the MDCKII Cell Monolayer. To examine whether FEX is a substrate of hBSEP and hBCRP, transcellular transport of 5 μM FEX across the MDCKII monolayer was determined in hOATP1B3/hBSEP and hOATP1B3/hBCRP double transfectants. The basal-to-apical transport of FEX was approximately 2.6 times greater than that in the opposite direction in hOATP1B3/hBSEP double transfectants (Fig. 1D), whereas the difference in each direction of transport of FEX was no more than 2-fold in control cells and hOATP1B3 and hBSEP single transfectants (Fig. 1, A–C). The difference in each direction of transport of FEX was no more than 2-fold in control cells, and hOATP1B3, hBCRP, and hOATP1B3/hBCRP transfectants (Fig. 1, E–H). On the other hand, the basal-to-apical transport of 0.1 μM pitavastatin, a bisubstrate of hOATP1B3 and hBCRP (Hirano et al., 2005b), was approximately 2.6 and 2.9 times greater than that in the opposite direction in transfectants expressing hBCRP and hOATP1B3/hBCRP (Fig. 1, K and L), respectively, whereas the difference in each direction of transport of pitavastatin was no more than 2-fold in control cells and hOATP1B3 transfectants (Fig. 1, I and J).

ATP-Dependent Transport of FEX by hBSEP and rBsep. To examine the substrate specificity of hBSEP and rBsep for FEX, membrane vesicles were prepared from HEK293 cells either infected with recombinant adenovirus harboring hBSEP, rBsep, or GFP cDNA. The uptake clearance of 0.1 μM TC as a positive control by hBSEP and rBsep after a 2-min incubation at 37°C in the presence of ATP or AMP was 788 and 43.6 μl/mg protein (hBSEP; mean, $n = 2$), 378 and 43.3 μl/mg protein (rBsep; mean, $n = 2$), respectively. The time-dependent uptake of 10 μM FEX by hBSEP- and rBsep-enriched membrane vesicles is shown in Fig. 2, A

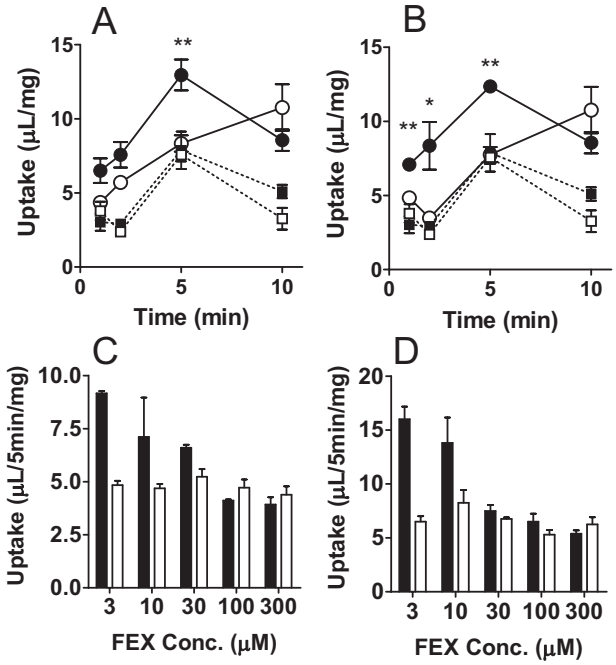

Fig. 2. The uptake of FEX in the membrane vesicles prepared from hBSEP- (A and C) and rBsep- (B and D) expressing HEK293 cells. A and B, the uptake of 10 μM FEX by hBSEP (A) and rBsep (B) for 5 min was examined at 37°C in the buffer containing 5 mM ATP (closed symbols) or AMP (open symbols). Circles and squares represent the uptake in hBSEP- (A) or rBsep- (B) and GFP-enriched membrane vesicles, respectively. C and D, the concentration-dependent uptake of FEX by hBSEP (C) and rBsep (D) was examined at 37°C in the medium containing 5 mM ATP (closed columns) or AMP (open columns). Each point and vertical bar represent the mean ± S.E. ($n = 3$). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols. *, $p < 0.05$; **, $p < 0.01$.

TABLE 2

Pharmacokinetic parameters of FEX during constant intravenous infusion into FVB mice ($n = 5$) and Mrp2$^{-/-}$ mice ($n = 6$)

Data represent the mean ± S.E. ($n = 5$ or 6). The meanings of these parameters are explained under *Materials and Methods*.

Parameters	FVB Mice ($n = 5$)	Mrp2$^{-/-}$ Mice ($n = 6$)
C_{ss} (nM)[a]	414 ± 54	597 ± 49*
$CL_{tot,plasma}$ (ml/min/kg b.wt.)	29.8 ± 3.9	20.1 ± 1.4*
$CL_{bile,plasma}$ (ml/min/kg b.wt.)	9.48 ± 0.82	3.38 ± 0.38**
$CL_{bile,liver}$ (ml/min/kg b.wt.)	0.250 ± 0.053	0.198 ± 0.022
V_{bile} (nmol/min/kg b.wt.)	3.71 ± 0.32	1.93 ± 0.14**
Bile flow rate (μl/min/kg b.wt.)	64.0 ± 7.1	53.1 ± 4.6
$K_{p,liver}$	40.7 ± 6.8	16.2 ± 2.0*
$CL_{urine,p}$ (ml/min/kg b.wt.)	16.9 ± 2.3	15.2 ± 3.2
V_{urine} (nmol/min/kg b.wt.)	6.59 ± 0.91	8.66 ± 1.79
GFR (ml/min/kg b.wt.)	17.3 ± 0.9	17.7 ± 2.5
$K_{p,kidney}$	22.7 ± 4.4	21.8 ± 3.1
$K_{p,brain}$	0.0183 ± 0.0028	0.0190 ± 0.0043

GFR, glomerular filtration rate.

[a] Corrected steady-state plasma concentration at the infusion rate of 700 nmol/h/kg.

* $p < 0.05$

** $p < 0.01$.

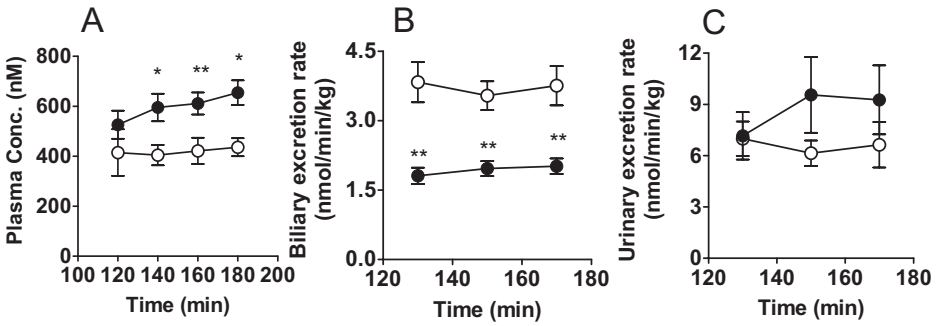

Fig. 3. Plasma concentration, biliary excretion rate, and urinary excretion rate of FEX during constant intravenous infusion into FVB mice and Mrp2$^{-/-}$ mice. The plasma concentration (A), biliary excretion rate (B), and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into FVB mice (○) and Mrp2$^{-/-}$ mice (●). Each point and vertical bar represent the mean ± S.E. (FVB mice, $n = 5$; Mrp2$^{-/-}$ mice, $n = 6$). *, $p < 0.05$; **, $p < 0.01$.

and B. The uptake of FEX was significantly stimulated by ATP in membrane vesicles prepared from hBSEP- and rBsep-enriched cells but not in those from HEK293 cells infected with GFP cDNA-harboring recombinant adenovirus. The concentration-dependent uptake of FEX is shown in Fig. 2, C and D. The uptake clearance in the presence of ATP was saturated in hBSEP- and rBsep-enriched membrane vesicles. However, the Michaelis constant (K_m) could not be evaluated because we could not measure the uptake clearance at less than 3 μM because this was lower than the detection limit.

Steady-State Pharmacokinetics of FEX in Wild-Type FVB and Mrp2$^{-/-}$ Mice. Although the previous study suggests that rMrp2 is not involved in the biliary excretion of FEX, there may be a species difference in the mechanism of FEX biliary excretion between rats and mice. Therefore, intravenous constant infusion into wild-type FVB and Mrp2$^{-/-}$ mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady state in wild-type FVB and Mrp2$^{-/-}$ mice are shown in Fig. 3. The pharmacokinetic parameters are summarized in Table 2. The plasma concentrations of FEX reached steady state within 120 min during the constant infusion to both strains of mice (Fig. 3A). The C_{ss} in Mrp2$^{-/-}$ mice significantly increased, and the $CL_{tot,plasma}$ significantly decreased compared with the values for FVB mice ($p < 0.05$). The $CL_{bile,plasma}$ and $K_{p,liver}$ in Mrp2$^{-/-}$ mice significantly decreased compared with that for FVB mice ($p < 0.01$), whereas the $CL_{bile,liver}$ in Mrp2$^{-/-}$ mice slightly decreased by 20% in comparison with FVB mice, although the difference was not statistically significant. There were no statistically significant differences in the other parameters.

ATP-Dependent Transport of FEX by hMRP3 and hMRP4. To examine whether sinusoidal efflux transporters hMRP3 and hMRP4 can accept FEX as a substrate, membrane vesicles were prepared from HEK293 cells infected with recombinant adenovirus harboring hMRP3, hMRP4, or GFP cDNA. As a positive control, the uptake of 0.1 μM EG by hMRP3 and hMRP4 after a 2-min incubation at 37°C in the presence of ATP or AMP was 298 and 3.26 μl/mg protein (hMRP3; mean, $n = 2$) and 170 and 5.17 μl/mg protein (hMRP4; mean, $n = 2$), respectively. The time-dependent uptake of 10 μM FEX by hMRP3- and hMRP4-enriched membrane vesicles is shown in Fig. 4, A and B. The uptake of FEX was significantly stimulated by ATP in membrane vesicles prepared from only hMRP3-expressing cells but not in those from hMRP4- and GFP-expressing cells. The concentration-dependent uptake of FEX is shown in Fig. 4C. The uptake clearance in the presence of ATP was saturated in hMRP3-enriched membrane vesicles. However, the Michaelis constant (K_m) could not be evaluated because we could not measure the uptake clearance at less than 3 μM because this was lower than the detection limit.

Steady-State Pharmacokinetics of FEX in Wild-Type FVB Mice and Mrp3$^{-/-}$ Mice. To investigate the effect of mMrp3 on the pharmacokinetics of FEX in vivo, intravenous constant infusion into Mrp3$^{-/-}$ mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady state in wild-type FVB and Mrp3$^{-/-}$ mice are shown in Fig. 5, and the pharmacokinetic parameters are summarized in Table 3. The plasma concentrations of FEX reached steady-state within 120 min during the constant infusion to both strains of mice (Fig. 5A). The C_{ss} in Mrp3$^{-/-}$ mice signif-

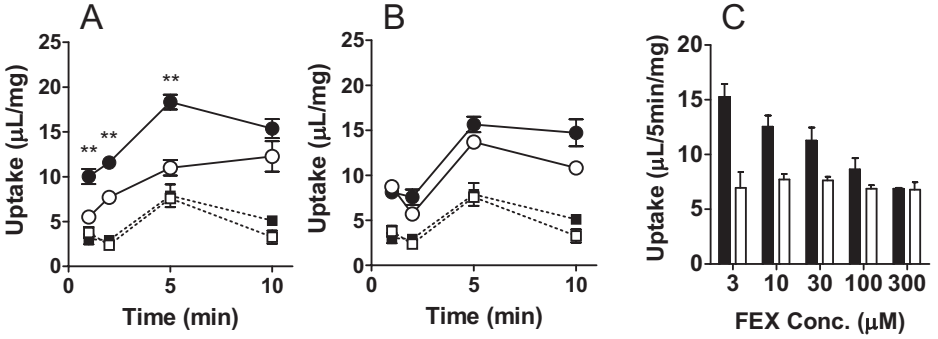

Fig. 4. The uptake of FEX in the membrane vesicles prepared from hMRP3- (A and C) and hMRP4- (B) expressing HEK293 cells. A and B, the uptake of 10 μM FEX by hMRP3 (A) and hMRP4 (B) was examined at 37°C in the medium containing 5 mM ATP (closed symbols) or AMP (open symbols). Circles and squares represent the uptake in hMRP3- (A) or hMRP4- (B) and GFP-enriched membrane vesicles, respectively. C, the concentration-dependent uptake of FEX by hMRP3 was examined at 37°C in the medium containing 5 mM ATP (closed columns) or AMP (open columns). Each point and vertical bar represent the mean ± S.E. ($n = 3$). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols. **, $p < 0.01$.

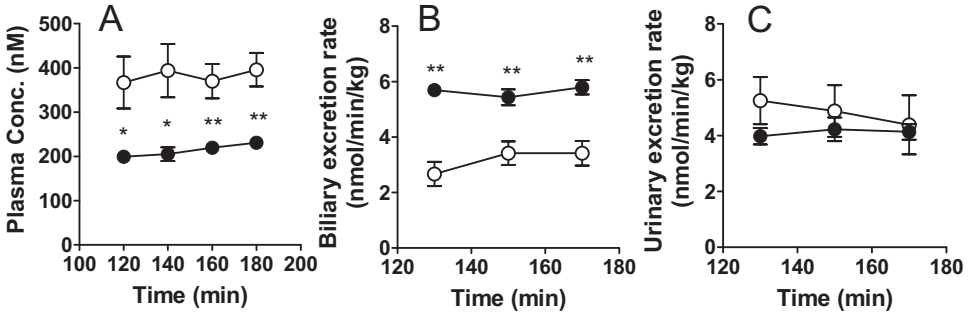

Fig. 5. Plasma concentration, biliary excretion rate, and urinary excretion rate of FEX during constant intravenous infusion into FVB mice and Mrp3$^{-/-}$ mice. The plasma concentration (A), biliary excretion rate (B), and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into FVB mice (○) and Mrp3$^{-/-}$ mice (●). Each point and vertical bar represent the mean ± S.E. (FVB mice, $n = 7$; Mrp3$^{-/-}$ mice, $n = 6$). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols. *, $p < 0.05$; **, $p < 0.01$.

icantly decreased and the $CL_{tot,plasma}$ significantly increased compared with that in FVB mice ($p < 0.01$). The $CL_{bile,plasma}$ and $K_{p,liver}$ in $Mrp3^{-/-}$ mice significantly increased compared with that in FVB mice ($CL_{bile,plasma}$, $p < 0.01$; $K_{p,liver}$, $p < 0.05$) and the $CL_{bile,liver}$ in $Mrp3^{-/-}$ mice significantly increased ($p < 0.01$). There were no statistically significant differences in the other parameters.

Steady-State Pharmacokinetics of FEX in Wild-Type C57BL/6 Mice and $Mrp4^{-/-}$ Mice. To investigate the effect of mMrp4 on the pharmacokinetics of FEX in in vivo, intravenous constant infusion into $Mrp4^{-/-}$ mice was performed. The plasma concentration, biliary excretion, and urinary excretion of FEX at steady-state in wild-type C57BL/6 and $Mrp4^{-/-}$ mice are shown in Fig. 6 and the pharmacokinetic parameters are summarized in Table 4. The plasma concentrations of FEX reached steady-state within 120 min during the constant infusion to both strains of mice (Fig. 6A). There were no statistically significant differences in any of the evaluated parameters.

LUI Study in Wild-Type FVB Mice and $Mrp3^{-/-}$ mice. The $CL_{bile,plasma}$ and $K_{p,liver}$ in $Mrp3^{-/-}$ mice significantly increased compared with that in FVB mice as mentioned previously. One of the possible reasons is the increase in the uptake clearance of FEX in $Mrp3^{-/-}$ mice. Therefore, we measured the initial uptake clearance of FEX in $Mrp3^{-/-}$ mice and FVB mice by using LUI experiment. After FEX (10 nmol/kg of body weight) was injected into the portal vein of wild-type FVB mice and $Mrp3^{-/-}$ mice, the hepatic extraction ratio was calculated. There was no significant difference in the extraction ratio between the FVB mice (0.885 ± 0.014; mean ± S.E., $n = 3$) and $Mrp3^{-/-}$ mice (0.885 ± 0.022; mean ± S.E., $n = 3$).

Relative Expression of Oatps, Mrps, Bcrp, Bsep, and Multidrug and Toxin Compound Extrusion 1 in the Liver, Bile Flow Rate, and Biliary Excretion of Total Bile Acids and GSH in Wild-Type FVB and $Mrp3^{-/-}$ Mice. The various kinetic parameters of FEX and the bile flow rate were changed in the $Mrp3^{-/-}$ mice. It is possible that the expression levels of hepatic transporters are different between $Mrp3^{-/-}$ and FVB mice. Therefore, the hepatic mRNA and protein expression levels of the transporters involved in drug transport were compared between FVB and $Mrp3^{-/-}$ mice using real-time quantitative PCR and Western blot analyses (Table 5 and Fig. 7). The no expression of Mrp3 mRNA and protein was confirmed in $Mrp3^{-/-}$ mice. The mRNA levels of mOatp1b2 and mouse multidrug and toxin compound extrusion 1 (mMate1) significantly decreased in $Mrp3^{-/-}$ mice ($p < 0.05$), whereas mBcrp significantly increased ($p < 0.05$; Table 5). However, these differences were no more than 2-fold. There were no statistically significant differences in the mRNA levels of the other transporters. The protein expression levels of the transporters in the crude membrane fraction normalized by the expression level of β-actin were also evaluated. There were less than 2-fold differences in the protein levels of mMrp2, mMrp4, mBsep, mMdr1, and mBcrp (Fig. 7). Because it is generally accepted that the bile flow rate depends on the biliary excretion of GSH and bile acids, the bile flow rate and biliary excretion of total bile acids and GSH were examined in wild-type FVB mice and $Mrp3^{-/-}$ mice and summarized in Table 5. The bile flow rate significantly increased in $Mrp3^{-/-}$ mice compared with FVB mice. Although the excretion rate of GSH in $Mrp3^{-/-}$ mice was approximately 1.3 times higher than that in FVB mice, the efflux clearance based on liver concentration in $Mrp3^{-/-}$ mice was not significantly different from that in FVB mice. And there is no statistically significant difference in the biliary excretion of total bile acids between FVB mice and $Mrp3^{-/-}$ mice.

TABLE 3

Pharmacokinetic parameters of FEX during constant intravenous infusion into FVB mice ($n = 7$) and $Mrp3^{-/-}$ mice ($n = 6$)

Data represent the mean ± S.E. ($n = 6$ or 7). The meanings of these parameters are explained under *Materials and Methods*.

Parameters	FVB Mice ($n = 7$)	$Mrp3^{-/-}$ Mice ($n = 6$)
C_{ss} (nM)[a]	382 ± 46	214 ± 11**
$CL_{tot,plasma}$ (ml/min/kg b.wt.)	33.6 ± 3.8	56.1 ± 3.1**
$CL_{bile,plasma}$ (ml/min/kg b.wt.)	9.32 ± 1.01	25.9 ± 1.1**
$CL_{bile,liver}$ (ml/min/kg b.wt.)	0.235 ± 0.025	0.495 ± 0.023**
V_{bile} (nmol/min/kg b.wt.)	3.17 ± 0.27	5.64 ± 0.19**
Bile flow rate (μl/min/kg b.wt.)	53.7 ± 3.4	88.6 ± 7.4*
$K_{p,liver}$	37.9 ± 3.4	48.0 ± 2.1*
$CL_{urine,p}$ (ml/min/kg b.wt.)	14.2 ± 2.3	19.0 ± 1.8
V_{urine} (nmol/min/kg b.wt.)	4.84 ± 0.91	4.11 ± 0.30
GFR (ml/min/kg b.wt.)	15.1 ± 2.1	19.9 ± 1.8
$K_{p,kidney}$	23.5 ± 2.3	24.8 ± 2.1
$K_{p,brain}$	0.0169 ± 0.0016	0.0171 ± 0.0021

GFR, glomerular filtration rate.

[a] Corrected steady-state plasma concentration at the infusion rate of 700 nmol/h/kg.

* $p < 0.05$

** $p < 0.01$.

Discussion

In the present study, we examined which hepatic efflux transporters can recognize FEX as a substrate by using transporter-expressing cells and membrane vesicles. We also investigated the importance of Mrp2, Mrp3, and Mrp4 in the in vivo pharmacokinetics of FEX by using the corresponding knockout mice.

The basal-to-apical transport of FEX was larger than the apical-to-basal transport in the hOATP1B3/hBSEP double transfectant but not in the hOATP1B3/hBCRP double trans-

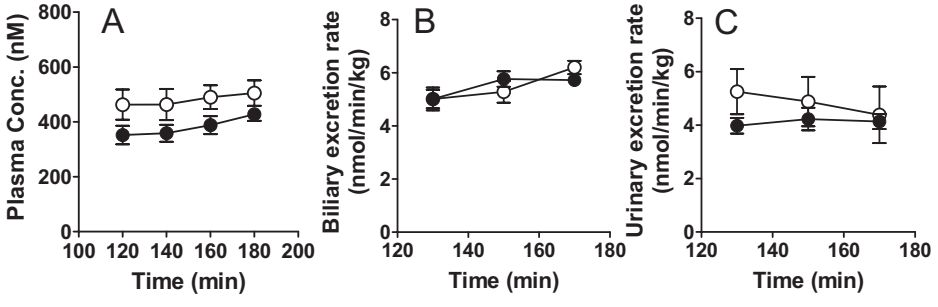

Fig. 6. Plasma concentration, biliary excretion rate, and urinary excretion rate of FEX during constant intravenous infusion into C57BL/6 mice and $Mrp4^{-/-}$ mice. The plasma concentration (A), biliary excretion rate (B), and urinary excretion rate (C) of FEX were determined during constant intravenous infusion into C57BL/6 mice (○) and $Mrp4^{-/-}$ mice (●). Each point and vertical bar represent the mean ± S.E. (C57BL/6 mice, $n = 4$; $Mrp4^{-/-}$ mice, $n = 3$). Where no vertical bars are shown, the S.E. values were contained within the limits of symbols.

fectant (Fig. 1). Moreover, ATP-dependent uptake of FEX was observed in hBSEP- and rBsep-enriched membrane vesicles (Fig. 2). These results indicate that FEX is a substrate of hBSEP and rBsep but not hBCRP. This result is consistent with a previous in vivo result demonstrating that the absence of mBcrp1 did not change its biliary excretion. We hypothesized previously that efflux transporters other than rMrp2, mMdr1, and mBcrp1 contribute to the biliary excretion of FEX (Tahara et al., 2005). It is interesting that BSEP could be a potential candidate transporter for its biliary excretion. BSEP is generally recognized as an efflux transporter for bile acids. However, recent studies have revealed that BSEP can also transport nonbile acids such as vinblastine and pravastatin (Lecureur et al., 2000; Hirano et al., 2005a). Further investigations to clarify the contribution of BSEP to the biliary excretion of drugs will be of interest.

Although previous results indicated a minor role of rMrp2 in the biliary excretion of FEX in rats (Tahara et al., 2005; Tian et al., 2008), species difference in the contribution of Mrp2 might exist between rats and mice. Therefore, to clarify the contribution of mMrp2 to FEX excretion, an in vivo infusion study was carried out using wild-type mice and Mrp2$^{-/-}$ mice (Fig. 3 and Table 2). The $CL_{bile,plasma}$ in the Mrp2$^{-/-}$ mice was approximately one third of that in the FVB mice, whereas the $CL_{bile,liver}$ in the Mrp2$^{-/-}$ mice was only 20% lower than that in the FVB mice. The $K_{p,liver}$ in the Mrp2$^{-/-}$ mice was much lower than that in the FVB mice. These results indicate that mMrp2 plays a limited role in the biliary excretion of FEX in mice, and unknown transporter(s) other than mMdr1, mMrp2, and mBcrp1 is involved in FEX transport across the canalicular membrane. The possible reason of the great decrease in the $CL_{bile,plasma}$ in the Mrp2$^{-/-}$ mice was a decrease in the hepatic uptake clearance and/or an increase in the sinusoidal efflux clearance from the liver to the blood. The expression levels of mMrp3 and mMrp4 in the Mrp2$^{-/-}$ mice are increased compared with wild-type mice, whereas no change in the expression levels of mOatp transporters in the liver was observed (Chu et al., 2006; Vlaming et al., 2006). Therefore, the increase in the sinusoidal efflux of FEX was probably caused by the increase in the expression of mMrp3 and/or mMrp4. While this article was under review, Tian et al. (2008) published the interesting findings in which biliary excretion clearance of FEX based on the unbound hepatic concentration decreased by more than 50%, and its hepatic concentration also considerably decreased in Mrp2$^{-/-}$ mice (Tian et al., 2008). Although the reason for the difference in the quantitative contribution of mMrp2 between previous study (in situ perfusion) and current study (in vivo pharmacokinetics) remains unclear, these results suggest that mMrp2 may be partly involved in its biliary excretion and its sinusoidal efflux considerably increases in Mrp2$^{-/-}$ mice.

To examine the involvement of MRP3 and MRP4 in the sinusoidal efflux of FEX, in vitro uptake studies using hMRP3- and hMRP4-enriched membrane vesicles and in vivo infusion studies using Mrp3$^{-/-}$ and Mrp4$^{-/-}$ mice were performed. ATP-dependent uptake of FEX was only observed in

TABLE 4

Pharmacokinetic parameters of FEX during constant intravenous infusion into C57BL/6 mice (n = 4) and Mrp4$^{-/-}$ mice (n = 3)

Data represent the mean ± S.E. (n = 3 or 4). The meanings of these parameters are explained under *Materials and Methods*.

Parameters	C57BL/6 Mice (n = 4)	Mrp4$^{-/-}$ Mice (n = 3)
C_{ss} (nM)[a]	480 ± 49	382 ± 29
$CL_{tot,plasma}$ (ml/min/kg b.wt.)	25.5 ± 2.9	31.1 ± 2.2
$CL_{bile,plasma}$ (ml/min/kg b.wt.)	10.9 ± 1.5	15.3 ± 1.1
$CL_{bile,liver}$ (ml/min/kg b.wt.)	0.369 ± 0.033	0.454 ± 0.025
V_{bile} (nmol/min/kg b.wt.)	5.50 ± 0.26	5.50 ± 0.18
Bile flow rate (μl/min/kg b.wt.)	71.1 ± 11.1	76.6 ± 2.2
$K_{p,liver}$	28.3 ± 3.7	29.5 ± 0.4
$CL_{urine,p}$ (ml/min/kg b.wt.)	14.7 ± 1.3	17.2 ± 3.1
V_{urine} (nmol/min/kg b.wt.)	7.69 ± 0.80	6.13 ± 0.85
GFR (ml/min/kg b.wt.)	13.6 ± 1.2	12.8 ± 2.4
$K_{p,kidney}$	15.6 ± 1.5	15.6 ± 1.7
$K_{p,brain}$	0.0210 ± 0.0015	0.0210 ± 0.0021

GFR, glomerular filtration rate.
[a] Corrected steady-state plasma concentration at the infusion rate of 700 nmol/h/kg.
* $p < 0.05$
** $p < 0.01$.

TABLE 5

Comparison of mRNA levels of various transporters expressed in mouse liver, bile flow rate, excretion rate, and efflux clearance based on the liver concentration of GSH and total bile acids between FVB mice (n = 3) and Mrp3$^{-/-}$ mice (n = 3)

Data represent the mean ± S.E. (n = 3). The meanings of these parameters are explained under *Materials and Methods*.

Parameters	FVB Mice	Mrp3$^{-/-}$ Mice
mRNA expression level normalized by the expression level of mGAPDH		
mOatp1a1	5.20 ± 0.63	5.66 ± 0.34
mOatp1a4	1.44 ± 0.17	1.42 ± 0.20
mOatp1b2	4.35 ± 0.12	3.02 ± 0.43*
mMrp3	1.03 ± 0.21	N.D.
mMrp4	0.0460 ± 0.0243	0.0255 ± 0.0032
mMrp2	18.8 ± 2.2	17.8 ± 1.73
mMdr1a	0.431 ± 0.064	0.231 ± 0.006
mMdr1b	0.319 ± 0.061	0.356 ± 0.117
mBcrp	0.610 ± 0.064	0.796 ± 0.018*
mBsep	11.3 ± 1.5	10.7 ± 1.1
mMate1	0.779 ± 0.038	0.620 ± 0.042*
Bile flow rate (μl/min/kg)	51.9 ± 8.6	81.1 ± 3.5*
Hepatic GSH concentration (mM)	4.27 ± 0.75	5.62 ± 0.13
GSH excretion rate (nmol/min/kg)	231 ± 37	300 ± 54
GSH efflux clearance (μl/min/kg)	54.8 ± 3.3	53.3 ± 9.1
Total bile acids excretion rate (μmol/min/kg)	4.21 ± 0.64	3.81 ± 0.28
Total bile acids efflux clearance (μl/min/kg)	498 ± 67	486 ± 28

N.D., not detected.
* $p < 0.05$.

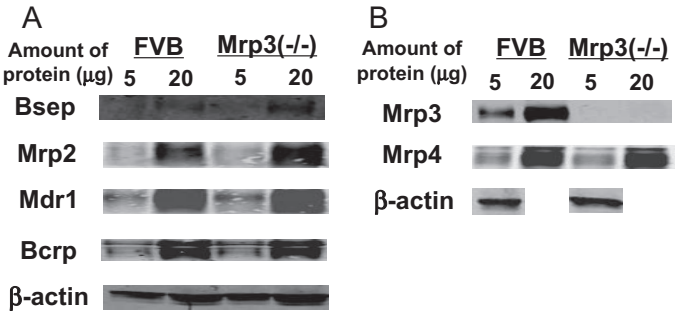

Fig. 7. Comparison of the protein expression levels of various transporters expressed in the crude membrane of mouse liver between FVB mice and Mrp3$^{-/-}$ mice using Western blot analyses. The expression levels of the efflux transporters expressed in bile canalicular membrane (A) and sinusoidal membrane (B) in the hepatic crude membrane fraction prepared from five FVB mice and Mrp3$^{-/-}$ mice were determined by Western blot analyses. β-Actin was used for the normalization of the expression level of each transporter.

hMRP3-enriched vesicles but not hMRP4- and GFP-enriched vesicles, indicating that FEX is a substrate of hMRP3 but not hMRP4 (Fig. 4). Until now, methotrexate and etoposide were the only unconjugated drugs reported to be transported by hMRP3 (Zelcer et al., 2001; Zeng et al., 2001). The identification of FEX as an MRP3 substrate suggests that it is worthwhile to check whether other drugs are substrates of MRP3. In in vivo kinetic analyses, the $CL_{bile,plasma}$, $CL_{bile,liver}$, and $K_{p,liver}$ values of FEX in the $Mrp3^{-/-}$ mice were greater than those in the wild-type mice (Fig. 5 and Table 3), whereas there was no difference in the pharmacokinetic parameters between the wild-type and $Mrp4^{-/-}$ mice (Fig. 6 and Table 4). This result is difficult to explain, because if the increase in the $CL_{bile,plasma}$ was simply caused by the increase in the $CL_{bile,liver}$, the $K_{p,liver}$ value should be reduced in the $Mrp3^{-/-}$ mice, which is opposite to our results. To resolve this discrepancy, the increase in the uptake clearance and/or the decrease in the sinusoidal efflux clearance in $Mrp3^{-/-}$ mice should be considered.

To examine whether the uptake clearance of FEX was increased in the $Mrp3^{-/-}$ mice, the expression levels of the Oatp transporters in the liver and the extraction ratio of FEX estimated by the LUI experiment were compared between the wild-type mice and the $Mrp3^{-/-}$ mice. The expression levels of Oatp1a1, Oatp1a4, and Oatp1b2 in the $Mrp3^{-/-}$ mice were almost the same as or slightly lower than those in the wild-type mice (Table 5). The extraction ratio of FEX in $Mrp3^{-/-}$ mice was not different from that in wild-type mice in the LUI experiment. However, because FEX was highly extracted into the liver in both strains, and the hepatic uptake clearance was much larger than the blood flow rate, so the change of uptake clearance does not affect its extraction ratio. Therefore, unfortunately we cannot conclude that the uptake clearance of FEX was not different between the wild-type and $Mrp3^{-/-}$ mice from this experiment. However, we have not obtained any evidence indicating that the uptake clearance of FEX increased in the $Mrp3^{-/-}$ mice. We currently think that the increase in the $K_{p,liver}$ value was mainly caused by the decrease in the sinusoidal efflux by the absence of Mrp3 expression rather than the enhanced uptake in the $Mrp3^{-/-}$ mice. Moreover, it can be considered that the increase in the sinusoidal efflux clearance in the $Mrp2^{-/-}$ mice was mainly due to an increase in the Mrp3 expression on the sinusoidal membrane.

We were surprised to find that $CL_{bile,liver}$ increased in $Mrp3^{-/-}$ mice. It is difficult to explain why the efflux via the canalicular membrane was affected by Mrp3 on sinusoidal membrane. A significant increase in the bile flow rate was observed in $Mrp3^{-/-}$ mice in comparison with wild-type mice (Tables 3 and 5). It is generally accepted that the bile flow rate depends on the biliary excretion of GSH and bile acids, which are mainly excreted by Mrp2 and Bsep, respectively (Elferink and Groen, 2002), so it is possible that the functions of Mrp2 and Bsep were changed in the $Mrp3^{-/-}$ mice. Therefore, the mRNA and protein expression levels of Mrp2, Bsep, and the other efflux transporters expressed in the canalicular membrane were compared between the wild-type and $Mrp3^{-/-}$ mice. It was unexpected that the difference in the expression levels of all the transporters was no more than 2-fold (Table 5 and Fig. 7). In addition, to investigate whether the function of Mrp2 and Bsep was changed, the biliary excretion clearance based on the intrahepatic concentration of GSH and total bile acids was calculated. However, no significant difference in the clearance of both GSH and bile acids was observed (Table 5). On the other hand, the excretion rate and hepatic concentration of GSH in $Mrp3^{-/-}$ mice were slightly higher than those in wild-type mice (Table 5). Manautou et al. (2005) showed that hepatic GSH content in untreated $Mrp3^{-/-}$ mice was slightly higher than that in wild-type mice. Therefore, the increase of hepatic GSH synthesis in $Mrp3^{-/-}$ mice might lead to an increase in the bile flow rate after the increase of the excretion rate of GSH. Thus, mMrp2 and mBsep are not likely to contribute to the increase of $CL_{bile,liver}$ of FEX in $Mrp3^{-/-}$ mice, and unidentified transporter(s) may be involved in the excretion of FEX in mice. It is also possible that the increase in the excretion of GSH might result in an increase in the secretion of FEX because

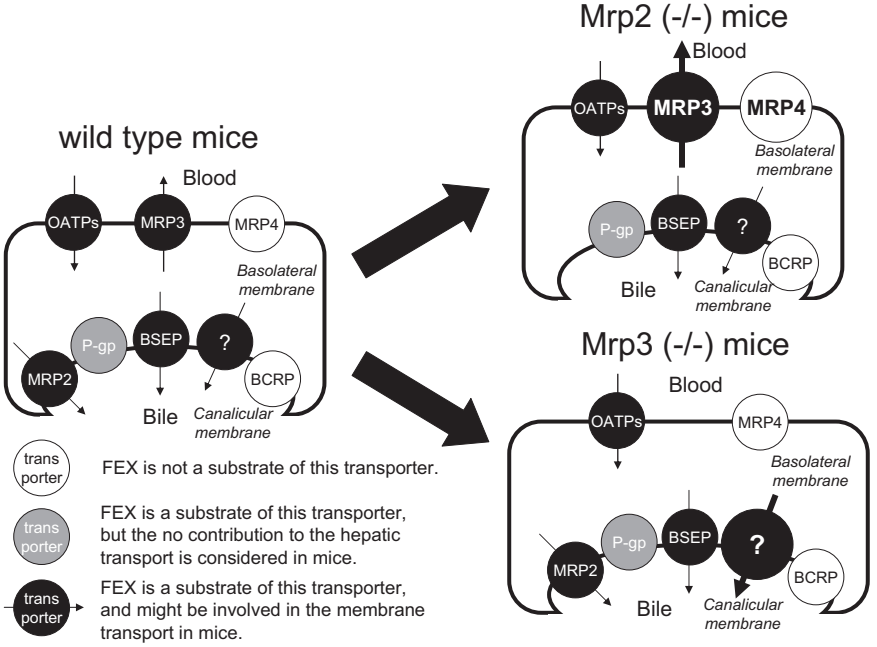

Fig. 8. Schematic diagrams of the proposed transport mechanisms of FEX in wild-type, $Mrp2^{-/-}$, and $Mrp3^{-/-}$ mice. FEX is a substrate of hOATPs, hMRP2, hMRP3, hBSEP, and P-gp in humans. In this figure, it is assumed that there is no difference in the substrate specificity of each transporter for FEX between humans and mice. In the $Mrp2^{-/-}$ mice, the expression levels of Mrp3 and Mrp4 are increased compared with those in the wild-type mice. In the $Mrp3^{-/-}$ mice, the unidentified transporter(s) may be increased compared with the wild-type mice.

GSH is known to stimulate the transport of substrates via Mrp2 (Van Aubel et al., 1999). Multiplicity of canalicular transporters has been proposed for the excretion of some compounds. For example, the excretion of bilirubin glucuronide across the canalicular membrane contains an ATP-independent transport system, which is stimulated by bicarbonate ion in addition to Mrp2 (Adachi et al., 1991). Further studies are required to clarify the multiple canalicular transport systems for xenobiotics.

The results obtained in the present and previous studies are summarized in Fig. 8. The in vitro studies clarified that FEX is a substrate of hBSEP/rBsep and hMRP3. In addition, the in vivo studies show that mMrp3 plays an important role in the sinusoidal efflux of FEX and consequently its pharmacokinetics, whereas mMrp2 plays a minor role in the canalicular excretion of FEX.

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